FINAL No.1 *REPORT ON*

CONTRACT NO DA 92-557-FEC-33136 03 6-C

INCLUSIVE DATES Dec. 21, 1959 *TO* Dec. 20, 1960

SUBJECT OF INVESTIGATION

EXPLANATION OF PATHOGENESIS OF JAPANESE B ENCEPHALITIS

AND

ESTABLISHMENT OF AVIRULENT STRAINS OF JAPANESE B ENCEPHALITIS VIRUS

RESPONSIBLE INVESTIGATOR

Akira OYA, M.D.
Chief, 4th Virus Division
Department of Virology & Rickettsiology
National Institute of Health, Tokyo, Japan

607 450

U.S. Army Research & Development Group (9852) (Far East)
Office of the Chief of Research and Development
United States Army
APO 343

EXPLANATION OF PATHOGENESIS OF JAPANESE B ENCEPHALITIS

AND

ESTABLISHMENT OF AVIRULENT STRAINS OF

JAPANESE B ENCEPHALITIS VIRUS

Akira Oya, M. D.

Chief of the 4th Virus Division,

Department of Virology & Rickettsiology,

National Institute of Health, Tokyo, Japan.

C O N T E N T

1. ISOLATION OF WILD JAPANESE B ENCEPHALITIS VIRUS STRAINS FROM MOSQUITOES AND FROM AUTOPSIED BRAINS OF PATIENTS

a. Purpose of Study

Japanese B encephalitis (JBE) virus is considered to be highly infective to man as well as domestic and wild animals. These facts were well demonstrated by the extensive survey of JBE antibody in the field (Bawell et al., 1950, Tigertt and Hammon, 1950, Buescher, 1956). However, it is also true that naturally infected animals scarcely show symptoms. In case of man, only a few among thousands was thought to manifest encephalitis (Southam, 1956). Little has been known concerning factors which may be involved in this divert pathogenesis. One of the purposes of this research is to investigate the pathogenesis of JBE from the side of virus particles.

To begin with this investigation in the laboratory, it was considered to be essential to determine strains of JBE virus employed in the experiment. Nakayama strain of JBE virus has been used mainly in the laboratory by previous investigators (Lennette and Koprowski, 1944, Imam and Hammon, 1957). However, being passaged through the adult mouse brain for more than hundred times, Nakayama strain is thought to be fixed to brain tissue artificially in the laboratory and seems unsuitable to use for the present experiment.

Therefore, attempts were made to isolate JBE virus without employment of the conventional method, i.e. intracerebral inoculation into mouse. Taking into account a hypothesis that there may be inhomogeny as regards the virulence among virus particles present in nature, JBE strains bearing various degree of virulence to the adult mice have been seeked. Especially, more neurotropic strain was expected to isolate from fatal human brain and less neurotropic strain was hoped to gain from naturally infected mosquitoes in higher probability.

b. Materials and Methods

(1) Cultivation of hamster kidney cells: Pairs of kidney of hybrid strain of golden hamsters were cut into pieces and trypsinized following Bodian's method (1956) with 0.2% trypsin. Primary cells were grown in square bottles using a medium containing 10% calf serum in 0.5% lactalbumin-Hanks solution (TCS). At its 5th to 7th day of cultivation, cells were again trypsinized, washed and transfered to tubes supplemented the same growth medium except employing JBE antibody-free calf serum (HCS). Cell numbers to be inoculated in a tube were adjusted to 1×10^5 cells per cc. After the following 2 to 3 days of incubation, monolayer cells developed were ready for use.

(2) Virus titration with hamster kidney (HK) cell tubes: Growth media were decanted off tubes and 0.9 cc of maintenance medium consisted of 4% horse serum (JBE antibody free) plus 0.5% lactalbumin-Hanks was added to each tube. A tube was inoculated with 0.1 cc of virus suspension. Four HK tubes were used per dilution. Inoculated tubes were observed for 7 days and 50% infective dose known by appearance of CP was calculated and called as

HKIU (hamster kidney infective unit).

(3) Preparation of mosquito-suspension for virus isolation: Culex tritaeniorhynchus which has been thought to be a main vector of JBE virus in Japan (Buescher 1956) were collected at live-stock pens at Gumma Prefecture in the summer 1959. 100 mosquitoes were pooled and emulsified with 2 cc of buffered saline containing 2% inactivated horse serum (HSS). Penicillin 500 units and streptomycin 500 gamma were added to avoid bacterial contamination. After clarification by low speed centrifugation, suspensions were dispensed in ampules, sealed and stored in a dry ice box.

(4) Inoculation into mice: Gpc strain and dd strain of albino mice were used. Suckling mice were used at their 2-4 days of ages and adult were at 28 to 32 ages. Intracerebral inoculation dose was 0.02 cc for suckling and 0.03 cc for adult mouse. Two tenth cc was used for intraperitoneal injection to the adult mouse and 0.05 cc to the suckling. One LD_{50} was termed as BLU for suckling and as ALU for adult mouse. Since some mice inoculated with less virulent virus recovered from the disease, AIU (adult mouse infective unit) was calculated as one 50% infective dose.

(5) Inoculation into eggs: Fertile hen's eggs were used after 24 hrs of incubation at 38°C. Inoculation and harvest were performed by the method described by Yoshino et al (1959). For virus titration 10 eggs were used per dilution. Inoculated eggs were observed for 7 days incubated at 37°C. OIU (one day egg infective unit) meant one LD_{50} calculated.

c. Experimental Results

(1) Attempt to isolate JBE virus from mosquitoes by use of cultivated hamster kidney cells and of one day old eggs. Suspensions of Culex tritaeniorhynchus caught in the field in the summer 1959 were tested for virus by i.c. inoculation into suckling mice in the previous investigation (Matsuyama et al, 1960). Virus-positive suspensions had been stored in a dry ice box at the commencement of the present investigation. Six pools were inoculated into HK tubes. Due to the toxicity encountered with the mosquito suspension to HK cells, stored suspension was diluted 5 times and its 0.1 cc was inoculated into 8 HK tubes. At their second blind passages, a strain of virus showing cytopathic effect on HK cells could be isolated. After 3 additional passages, CPE of this virus became distinct and it was eventally identified as JBE virus by neutralization test in HK tubes (Table 1). This strain was refered as JaGAr 01-HK strain.

Table 1. Neutralization tests with JaGAr 01-HK and JBE virus immune serum in HK cells.

The same mosquito suspensions were inoculated simultanously into 10 one day old eggs each expecting cease of development of the embryos as observed with the laboratory strain (Matumoto, 1957, Okuno, 1959). However, the attempt was failed being interfered by frequent occurence of bacterial or mycotic contamination.

(2) Infectivity of mosquito-borne virus to mice and eggs. Before doing detailed analysis of virulence of JaGAr 01-HK strain, susceptibilities of some hosts to this virus were determined. HK culture fluid infected with this virus at its 6th HK passage was titrated in HK tubes, one day eggs, suckling and adult mouse brains. Titers of the fluid obtained with 4 kinds of host were listed below in log.

HKIU (hamster kidney infective units) ...	5.7/cc
OIU (one day egg infective units)	4.4/cc
BLU (baby mice lethal units)	6.9/cc
ALU (adult mice lethal units) equaling to AIU (adult mice infective units)	6.0/cc

Four kinds of host were found to be all susceptive to this strain, being highest in suckling mouse brain and lowest in one day eggs. Titers obtained by HK cells and adult mouse brain were quite comparable with each other. In general, the pattern indicated here by JaGAr 01-HK strain seems quite similar to what had been obtained by mouse brain fixed human-borne Nakayama strain.

(3) Isolation and passage of JBE virus from brain by intraperitoneal inoculation to adult mice. Provided inhomogeny of JBE virus exsisting in nature, one can assume that the virus strain causing fatal outcome in man might bear more neurotropic property. Such a increased neurotropism would mean not only a capability to multiply in brain tissue, but a property to be able to induce encephalities when administered even by peripheral route. Therefore, an attempt was taken to isolate a strain from autopsied human brain by intraperitoneal infection into adult mouse. Further, highly neurotropic strain was ecpected to be selected by the repeated ip-brain passages.

Human brains from cases of JBE had been stored in a dry ice box. Brain was emulsified in HSS, centrifuged lightly and titrated with suckling mouse brain in pararell with adult mouse i.p. Fig. 1 shows the results obtained by two strains from two human brains.

Fig. 1. Mortality of adult mice infected with human borne virus by i.p. route of inoculation

As shown in Fig. 1, JBE virus from human brain caused death in adult mice even when inoculated with higher dilution of virus suspension. When virus was titrated in suckling mice i.c., only a few BLU was found to be enough to cause death in adult mice by i.p. inoculation.

However, i.p. virulence to the adult mice was not strong enough to induce 100% death even at a dilution containing more than 10^5 BLU. Then, it was hoped to select more virulent strain by successive ip-brain passages to the adult mice. The strain described was hereafter designated as JaTH 160. Accordingly, successive passages were done taking brain from a mouse, on one hand, showing symptom at the lowest dilution of the inoculum and from a

mouse, on the other, become sick at the highest dilution. Mortality of the adult mice at various passages of this kind was illustrated in Table 2.

Table 2. Mortality of adult mice infected with JaTH 160 strain at various passage levels.

It can be seen in Table 2 that virulence of JaTH 160 to adult mouse, as firstly observed in Fig. 1, has been well maintained until 6th passages. However, increased virulence hoped in the beginning of these passages seems not to be gained. Irregularity of death of the mice were still in the case at its 6th ip-brain passages.

d. Discussion

JBE virus exsisting in nature seems not to be strictly neurotropic since it mainly cause inapparent infection without any sign of involvement of central nervous system and since it was proven by the experiment that there was a period during which virus would multiply outside of central nervous system (Imam and Hammon, 1957). Recently more evidences have been accumulated to indicate that JBE virus can grow abundantly in non-nervous cells. (Scherer and Syverton, 1954. McCollum and Foley, 1957). Particularly, the use of one day eggs (Matumoto, 1957), hamster kidney cells (Kissling, 1957), porcine kidney cells (Lee et al, 1958) and chick embryo monolayer (porterfield, 1959) furnished new methods for titration of JBE virus. That means one can compare the infectivity of a strain of JBE virus to a host with that to another host, and eventually enables us to analyse inhomogeny, if any, of JBE virus concerning virulence to a selected host cell.

As described in the text, new strains of JBE virus were isolated by such a method that maintain a peculiar infectious property, if it exsist, i.e. to keep less neurotropic strain with non-nervous cell passage or to keep or exaggerate strongly neurotropic strain by serial ip-brain passages. These strains, in addition to avienized strain and mouse brain fixed strains already on hand, are hoped to be good materials to investigate the pathogenesis of JBE from the side of virus particles.

e. Summary

Strains of JBE virus were isolated from mosquitoes and from human autopsied brain materials. Hamster kidney cells were selected as a host for the former strain to maintain a hypothetical more pantropic nature and intraperitoneal-brain passages were performed for the latter to maintain or to exaggerate more neurotropic nature of virus particles. Mosquito-borne strain was found to be highly infective to eggs and mouse brain. Human-borne strain showed higher but irregular virulence to the adult mouse by peripheral inoculation. These 2 strains newly propagated were considered to be useful for the experimental investigation of pathogenesis of JBE.

2. AN ATTEMPT TO ISOLATE A MUTANT STRAIN OF JAPANESE B ENCEPHALITIS VIRUS LESS VIRULENT TO ADULT MICE

a. Purpose of Study

As the control method of Japanese B encephalities (JBE) prevalent in Asian countries, vaccination was considered to be the most possible tool and a devise of effective JBE vaccine has been longly desired. A formalinized vaccine made from infected mouse brain was considered to be effective not only by the results of laboratory experiment (Sabin et al, 1943) but also by field trials (Tigertt and Berge, 1957). Nevertheless, brain type vaccine has not been considered satisfactory due to a risk of possibility to induce allergic encephalitis to animals (Kabat et al, 1947). JBE vaccine employing infected chick embryo thus has been studied extensively, but results was not encouraging.

On the other hand, superiority of live vaccine has been increasingly emphasized for control of viral diseases. In case of JBE too, live vaccine seems quite hopeful for control measure.

There are two purposes on the attempt to isolate a less virulent mutant of JBE virus. One is for the investigation of pathogenesis of JBE from the point of virus particles. Another aims at establishment of a JBE strain being suitable to apply to man as a live vaccine.

b. Materials and Methods

(1) Virus: TOP strain of JBE virus, reported by Okuno (1959) to have resulted in some attenuated status after successive passages to one day eggs was employed in the experiment exculsively.

(2) Clone selection from virus suspension by HK tubes: Suitable dilution series of virus suspension were made in HSS and 30 to 32 HK tubes were inoculated with a dilution. Clonal virus suspension was taken from a HK tube showing definite CP at a dilution at which more than 75% of inoculated tubes were found to be CP-negative.

(3) Others: Described already in the first chapter.

c. Experimental Results

(1) Use of one day egg. TOP strain was further passaged through one day eggs hoping higher attenuation to the adult mouse brain. Dilutions of 10^{-3} or 10^{-4} of egg homogenate were inoculated into the following one day eggs. After 71 passages, TOP strain was titrated in one day egg, baby and adult mouse brains simultaneously. Comparative titer with each host is the following:

OIU (one day egg infective unit)	7.6 log/cc
BLU (baby mouse lethal unit)	8.2
ALU (adult mouse lethal unit)	5.0

It is obvious that TOP strain at its 71st passage still had decreased virulence to the adult mouse brain. However, the ratio OIU/ALU did not increase so far beyond 2.6 which was obtained at its 29th passage level by Okuno (1959). This fact might suggest that a possibility obtaining further attenuated virus by this method is less probable.

(2) Use of hamster kidney cells.

i) First experiment. Based on a hypothesis that the virulence to the nervous tissue of JBE strain might decrease by successive passages through non-nervous cells, hamster kidney (HK) cells has been adopted for host cells of TOP strain following one day egg cells. After 4 HK passages employing low dilution seeds, terminal dilution passages have been employed successively until 10th passage as illustrated in Fig. 2.

Fig. 2. Terminal dilution passages of TOP strain through HK cells.

At its 7th, 9th and 10th passage levels, tissue culture fluids were titrated with baby and adult mouse brains in pararell with HK cells. Results are summarized in Table 3.

Table 3. Degree of virulence to adult mice of TOP strain at various passage levels in HK cells.

Due to cases of occasional recovery of obviously sick adult mice, AIU (adult mouse infective unit) was calculated separately from ALU. Sickness of baby mouse was found to be definitely lethal. As indicated in Table 3, TOP strain grew in HK cells abundantly, HKIU (hamster kidney cell infective unit) of culture fluid showing 7.0 to 8.0 in log. Titers obtained in adult mouse brain were consistently less than those in HK cells. However, the ratio HKIU/ALU, which was considered to be an index for attenuation of neurovirulence of virus was still hanging about as low as 2.0 to 3.0.

This fact would mean that a large population of TOP virus particles grown in HK cells undergoes no change in neurovirulence. If it is the case, terminal dilution passage employed here in HK cells may be unadequate for the purpose of selecting less neurotropic virus particle. Therefore, the following passages were made using undiluted infected fluid as inocula considering a chance to pick up less virulent mutant which might be contained in the infected HK culture fluid in a very limited extent. After 20 additional undiluted passages, infected tissue culture fluid was titrated as previous. The result was quite stimulating in regard to the attenuation. Owing to the fact that only an occasional death was observed in adult mice inoculated i.c. with this infected fluid. Results were illustrated in Table 4.

Table 4. Mortality of adult mice inoculated with TOP strain passaged by undiluted seed for 20 times.

ii) Second experiment. Looking back the results of successive passages of TOP strain through one day eggs, it happened once that TOP showed a remarkable decreased titer in the adult mouse brain (1.8 in log) though its titer in one day eggs was as high as 7.0 in log at 51st passage level (Okuno, 1959). This remarkable difference has never been obtained when it was reexamined at 56th and 72nd passage levels and considered that the result at 51st passage had to be one of the exceptional one due to fluctuation of viral titration in eggs. However, a problem was remained to be solved that a much less neurotropic variant had been present, uncovered once, and disappeared rapidly during passages. Fortunately, egg homogenate at 51st passage was still stored in a dry ice box and examined for reproducibility of the previous result. As the consequence, this suspension did not induce disease in the adult mice even when its 10^{-1} dilution was inoculated i.c., while it retained a high titer both in one day eggs and in HK cells (7.5 in log). Encouraged with this fact, terminal dilution passages were again carried out starting from egg homogenate at 51st passage. After 54th egg passages, it was transfered to HK cells. Variation of neurovirulence are presented in Table 5.

Table 5. Mortality of adult mice inoculated with TOP strain during terminal dilution passages after 51st passage through one day eggs and HK cells.

As shown in the figure, remarkable decrease of ic virulence which had never been encountered was repeatedly demonstrated, though mortality of mice was still fluctuating.

(3) Clonal passages of less virulent variant. The fluctuation of mortality of the mice following inoculation from passage to passage may be due to, at least in part, the mixed population of the inocula consisted of viral particles of various degree of neurovirulence. Therefore, clonal analysis was attempted during a few passage levels for their variability of neurovirulence. At the same time, taking a clone showing the least virulence among clones obtained at each passage, clonal passages were undertaken. Table 6 demonstrates the events.

Table 6. Fate of adult mice inoculated with clones of TOP strain obtained during a few clonal passages.

It became apparent, as shown in the table, that some degree of fluctuation of mortality of mice was unavoidable even among a viral population purified by repeated clonalizations. This fact, in turn, suggests that mortality of mice is not simply controled by gene factor. Then, it is not surprising that the attempt to select of a virus strain of progressed attenuation was

not successful.

(4) Comparison of strain and sex of mice for succesptibility to virus. Since gpc strain of adult mouse was exclusively used for the present investigation, another strain dd was examined to see whether strain of mouse has some role in neurovirulence of the virus. On the other hand, a possibility was determined whether viral virulence depends upon sex of mice inoculated. Table 7. illustrates the results indicating no important effect of both factors.

Table 7. Effect of strain and sex of mice in regards to the susceptibility to ic-challenge of TOP strain.

d. Discussion.

In order to investigate pathogenesis of JBE, mouse was selected as an experimental host because of the reasons, that it was one of the most sensitive animals to JBE virus and it was one of the most easy, economical animals for use in the laboratory. Employing this host, virulence of JBE virus was firstly examined from the point of that to central nervous system, i.e. neurovirulence due to its outstanding feature. The idea of neurovirulence could be understood at present in mouse, on the one hand, to mean ability to produce encephalitis by peripheral infection (sc-virulence or ip-virulence for instance) and, on the other, to mean ability to produce encephalitis by direct inoculation into brain with virus. Accordingly, the attenuation of neurovirulence would mean inability in either case. In this experiment, a less virulent strain has been searched in the latter sense, namely select a strain not to induce fetal disease in mice by its direct inoculation into brain. Four week old mice was exclusively employed in the present investigation.

A mutant strain was consequently isolated as seen in the text to have a decreased neurovirulence to the adult mice. No symptom could be observed in the adult mice by peripheral inoculation with this strain. Only an irregular and occasional death could be observed when the virus was administered directly to brain in large amount. Several attempts including application of non-nervous cells, clonal passages were not fruitful to induce further attenuation. Only with undilution-passages, some progress seemed to be made. However, even in this case, no one knows what is happening during the passages. As a whole, its appearance seems to be quite incidental. Decreased neurovirulence of this strain seems to be partly controled by genetic factor, but its instability would suggest the involvement of some other mechanisms as indicated by the previous investigator (Sabin, 1941). On the immunogenicity of the present attenuated strain, an important property for the application to vaccine, some description will be made in latter chapter.

e. Summary.

One day eggs and cultivated hamster kidney cells were selected as the hosts to select much less neurovirulent variant from partially attenuated

TOP strain of JBE virus. Eventually, a strain was elucidated as highly attenuated one showing only a few death even when its concentrated suspension was administered into adult mouse brain. Its isolation was rather incidental than being intentionally induced.

3. COMPARISON OF SEVERAL JAPANESE B ENCEPHALITIS VIRUS STRAINS IN TERM OF VIRULENCE TO THE ADULT MICE

a. Purpose of Study

Our general purpose is to elucidate pathogenesis of Japanese B encephalitis (JBE) virus from the side of virus particles.
The purpose of the experiment described in this chapter is to know the events occuring in the adult mice infected with a variety of JBE virus strain, laboratory fixed or wild, of mosquito borne or human borne, changing the inoculation route.

b. Materials and Methods

(1) Hemagglutination-inhibition test (HI). To prepare hemagglutinating antigen and to conduct HI, the technique devised by Clarke and Casals (1958) was employed. Antibody produced by mouse infected with a strain of JBE virus was examined against the antigen of the same strain.

(2) JBE virus strains. The histories of JaTH 160, JaGAr 01-HK and TOP strains were already described. The infected adult mouse brain suspensions were used as seed of JaTH 160 and infected HK cell fluid was employed in cases of JaGAr 01-HK and TOP. Number of passage was described in the test. Nakayama (NIH) and Nakayama (Yakken) strains are classical, adult mouse brain fixed strains of unknown passage number. They were used as infected baby mouse brain suspensions. JaGAr 02 strain was isolated from mosquitoes caught in 1959 by ic-inoculation into suckling mice. Its infected suckling mouse brain suspension of 3rd passage was employed.

(3) Other materials and methods. Described already in the previous chapters.

c. Experimental Results.

(1) Lethality of mice by peripheral inoculation of several strains of Japanese B encephalitis virus. Four week old adult mice were inoculated i.p. with some strains of JBE and mortality was recorded during 3 weeks after the inoculation. The inocula were titrated simultaneously with suckling mouse brain to estimate infectious particles injected. Results were illustrated in Table 8.

Table 8. Mortality of adult mice following ip inoculation with some strains of JBE virus.

In brief, there seemed to be two groups of strains concerning ip-virulence. Nakayama strain of 2 different sources produced only a few death by the inoculation of a great number of virus particles and no death was observed in the mice inoculated with TOP. On the contrary, death could be induced by the inoculation of JaTH 160, JaGAr 01-HK and JaGAr 02 strains even at

dilutions containing small number of infecting particles although death had never been definite at any dilutions. The difference of the attitude of 2 groups are most remarkable when two extremes, TOP and JaTH 160 are compared.

(2) Peripheral infectivity of strains of Japanese B encephalitis virus to mice. A question arose whether the mice being apparently normal during 3 weeks' observation were infected or not after the ip inoculation of virus. To answer the question, survived mice were bled at the end of the observation period (3 weeks) and sera pooled for each dilution or serum of the individual mouse separately were examined for presence of HI-antibody. It was thought that the mouse was infected if antibody was detected in its serum. Since it was no doubt that dead mouse was due to the infection, infection of the mouse was determined either by death or by detection of HI-antibody. As indicated in Fig. 3, almost all mice inoculated with significant amount of JaTH 160 strain of virus were found to be infected, infecting titer being close to ic titer obtained in suckling mice. On the other hand, antibody response could be seen only in a very limited number of mice infected with a large number of baby mouse infective unit in cases of both Nakayama strains. No mouse responded by the ip inoculation of TOP strain.

Fig. 3. Peripheral infectivity of some strains of JBE virus to the adult mice.

d. Discussion

Present results revealed differences of virulence to the adult mice among strains of JBE virus following ip or ic administration of virus. By ic inoculation, only with attenuated TOP strain, decreased virulence could be observed in term of mortality and in other cases higher mortality was always encountered. However, it is worthwhile to note that decreased virulence does not necessarily mean incapability of virus to multiply in brain tissue. Good evidence is in our hand with TOP strain to indicate its slow multiplication in adult mouse brain even after ic inoculation with higher dilution of virus suspension.

By the peripheral inoculation, at least two types could be observed among JBE strains tested. In one type, not only death but infection resulted in a very limited extent, i.e. merely when the mouse was inoculated with a great number of virus particles estimated by ic-inoculation into suckling mice. No evidence of infection could be seen so far in the rest of mice. While, definite infection or death occurred in another type of strain even when inoculated with a small amount of virus particle. The former type, so far as our limited experiment is concerned, seems to correspond to laboratory fixed strain, i.e. Nakayama and TOP and the latter no newly isolated, wild strains. So long as tested at present, no difference could be shown between mosquito-borne and human-borne wild strains. In brief, the following scheme might be drawn as regards the

virulence of various strains of JBE virus to the adult mice.

Table 9. Scheme of strain differences of JBE virus in regard to virulence to the adult mouse.

This kind of scheme will be useful for the further investigation of pathogenesis of JBE and as a guide to devise a new method to select an avirulent mutant of JBE virus. For instance, provided that one can seek for avirulent strain for vaccine employing adult mouse as an experimental host, a hypothetical variant No. 2 (See Table 9) would be most hopeful strain, though there is little possibility to induce such a variant from strains already in our hand. However, another type of attenuated variant No. 1 appears much easier to obtain from wild type of JBE strains because of the instability of peripheral mortality of the latter, and there may be more probability to gain No. 2 variant through No. 1 by selecting virus particles having less affinity to brain tissue.

e. Summary

Strains of JBE virus of various isolation- and passage-histories were examined for virulence to the adult mouse. Mortality and infectivity of virus were recorded after the peripheral inoculation. The latter property was estimated by the detection of antibody 3 weeks after the inoculation. Consequently, laboratory strains were differentiated markedly from wild strains by this procedure, being the former less infective to the adult mouse by peripheral route of inoculation. Attenuated TOP strain was separable by the decreased ic-virulence from the other laboratory fixed strains

4. REFERENCES

Bawell, M. B., Deuel, R. E., Jr., Matumoto, M. and Sabin, A. B., (1950): Status and significance of inapparent infection with virus of Japanese B encephalitis in Japan in 1946. Am. J. Hyg., 51, 1-12.

Bodian, D. (1956): Simplified method of dispersion of monkey kidney cells with trypsin. Virolory, 2, 575-576.

Buescher, E. L. (1956): Arthropod-borne encephalitis in Japan and Southeast Asia. Am. J. Pub. Health, 46, 597-600.

Clarke, D. H. and Casals, J. (1958): Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med. & Hyg., 7, 561-573.

Imam, I. Z. E. and Hammon, W. McD. (1957): Immunologic response and pathogenesis of Japanese B infection in peripherally inoculated normal and cortisone treated hamsters. Proc. Soc. Exp. Biol. & Med., 95, 12-16.

Kabat, E. A., Wolf, A. and Bezer, A. E. (1947): The rapid production of acute disseminated encephalomyelitis in rhesus monkeys by injection of heterologous and homologous brain tissue with adjuvants. J. Exp. Med., 85, 117-130.

Kissling, R. E. (1957): Growth of several arthropod-borne viruses in tissue culture. Proc. Soc. Exp. Biol. & Med., 96, 290-294.

Lennette E. H. & Koprowski, H. (1944): Influence of age on the susceptibility of mice to infection with certain neurotropic viruses. J. Immunol., 49, 175-191.

Matumoto, M. (1957): Inoculation de l'oeuf incubé pendant vingt-quatre heures avec le virus d'encéphalite japonaise. Compt rend. Soc. Biol. 151, 1466-1470.

Matuyama et al (1960): Isolation of arbor viruses from mosquitoes collected at live-stock pens in Gumma Prefecture in 1959. Jap. J. Med. Sci. & Biol., in press.

Mc.Collum, R. W. and Foley, J. F. (1957): Japanese B encephalitis virus in tissue culture. Proc. Soc. Exp. Biol. & Med., 94, 556-560.

Okuno, T. (1959): Isolation and passage in one-day eggs of Japanese B encephalitis (JBE) virus resulting in attenuation of its mouse pathogenicity. Jap. J. Med. Sc. & Biol., 12, 71-78.

Porterfield, J. S. (1959): Plaque production with yellow fever and related arthropod-borne viruses. Nature, 183, 1069-1070.

Sabin, A. B. (1941): Constitutional barriers to involvement of the nervous system by certain viruses, with special reference to the role of nutrition.

Sabin, A. B., Duffy, C. E., Warren, J., Ward, R., Peck, J. L., Jr., and Ruchman, I. (1943): The St. Louis and Japanese B types of epidemic encephalomyelits. Development noninfective vaccines: Report of basic data. J. A. M. A., 122, 477-486.

Scherer, W. F. & Syverton, J. T. (1954): The viral range in vitro of a malignant human epithelial cell (strain HeLa, Gey). II. Studies with encephalitis viruses of the eastern, western, West Nile, St. Louis and Japanese B types. Am. J. Path. 30, 1075-1084.

Southam, C. M. (1956): Serologic studies of encephalitis in Japan. II. Inapparent infections by Japanese B encephalitis virus. J. Infect. Dis., 99, 163-169.

Tigertt, W. D. and Berge, T. O. (1957): Japanese B encephalitis. Am. J. Pub. Health, 47, 713-718.

Tigertt, W. D. and Hammon, W. M. (1950): Japanese B encephalitis: a complete review of experience on Okinawa 1945-1949. Am. J. Trop. Med., 30, 689-722.

Yoshino, K., Taniguchi, H. & Taniguchi, S. (1959): Titration of herpes simplex virus in one-day eggs by an improved inoculation method and its application to the serum neutralization test. J. Immunol., 83, 246-251.

5. ABSTRACT OF FINAL REPORT.

Special consideration was taken on sources and passage-histories of Japanese B encephalitis (JBE) virus strains to be used in the experiment before commencing of investigation on pathogenesis of JBE. Strains of JBE virus were isolated from mosquitoes and from human autopsied brain materials. Non-nervous cells, hamster kidney (HK) cells, were selected as a host for the former strain to maintain a hypothetical pantropic nature and intraperitoneal-brain passages were performed for the latter to maintain or to exaggerate more neurotropic nature of virus particles. On the other hand, efforts were made to pick up less virulent virus mutant to adult mouse brain. One day eggs and HK cells were adopted as the hosts to select less virulent mutants from partially attenuated TOP strain of JBE virus. Eventually, a strain came into the light as highly attenuated one showing only a scanty death even when its large amount was inoculated into adult mouse brain. In spite of several attempts, such a attenuated variant appear to have resulted incidentally rather than being intentionally induced.

In the meantime, strains of JBE virus of various histories were examined for virulence to the adult mouse. Mortality and infectivity of each strain were recorded after the peripheral inoculation. Infectivity was estimated by detection of HI-antibodies 3 weeks after the inoculation. Consequently, laboratory-fixed strains were differentiated from wild strains, being the former less infective to the adult mice by peripheral route of inoculation. The attenuated strain was deviated from other laboratory-fixed strains by the decreased intracerebral virulence.

Table 1. Neutralization tests with JaGAr 01-HK and JBE virus immune serum in HK cells.

Sera	Virus titer in log	Neutralization Index
Normal rabbit	7.0	-
**Nakayama immune mouse	4.5	2.5
*JaGAr 21 immune mouse	2.0	5.0

* Newly isolated JBE virus in 1959.

** Laboratory-fixed JBE virus strain.

Table 2. Mortality of adult mice infected with JaTH 160 strain at various passage levels.

Passage	Passage Dilution	Death of mice inoculated i.p.
IP-1	-	3/5 3/5 2/5 0/5 0/5 0/5 3.6 2.6 1.6 0.6 0.06 0.006
IP-2	-1	2/5 2/5 1/5 3/5 4/5 4/5 2/5 7.8 6.8 5.8 4.8 3.8 2.8 1.8
IP-3	-1	3/5 3/5 2/5 5/5 5/5 3/5 2/5 7.7 6.7 5.7 4.7 3.7 2.7 1.7
IP-4	-1	3/5 4/5 3/5 1/5 3/5 4/5 2/5 6.8 5.8 4.8 3.8 2.8 1.8 0.8
IP-5	-2	3/5 4/5 3/5 3/5 2/5 3/5 1/5 7.7 6.7 5.7 4.7 3.7 2.7 1.7
IP-6	-3	0/5 3/5 4/5 3/5 2/5 3/5 0/5 6.4 5.4 4.4 3.4 2.4 1.4 0.4
IP-2	-3	4/5 2/5 2/5 2/5 4/5 4/5 3/5 7.9 6.9 5.9 4.9 3.9 2.9 1.9
IP-3	-7	3/5 4/5 4/5 4/5 4/5 3/5 1/5 6.5 5.5 4.5 3.5 2.5 1.5 0.5
IP-4	-7	1/5 2/5 0/5 4/5 2/5 3/5 3/5 8.6 7.6 6.6 5.6 4.6 3.6 2.6

Numbers of upper line mean: Numerator, number of dead mice;
Denominator, number of inoculated mice.
Numbers of lower line mean BLU injected to mice, i.p.

Fig. 2. Terminal dilution passages of TOP strain at various passage levels in HK cells.

Passages of Inocula	Dilution of inoculum			
	10^{-5}	10^{-6}	10^{-7}	10^{-8}
HK-4	4 4 4 3	4 4 4 0	0 0 (3) 0	0 0 0 0
HK-5	4 4 0 0	0 0 (4) 0	0 0 0 0	0 0 0 0
HK-6	3 3 3 3	3 3 0 (3)	0 0 0 0	0 0 0 0
HK-7	4 4 4 4	4 0 (3) 4	0 0 0 0	0 0 0 0
HK-8	4 4 4 4	4 4 4 0	(4) 0 0 4	0 0 0 0
HK-9	4 4 4 4	3 0 0 4	0 0 0 0	0 0 0 0

* Note: Numbers mean grade of CP in a tube: 4, complete, 3, partial, 0, no destruction

Table 3. Degree of virulence to adult mice of TOP strain at various passage levels in HK cells.

Passages	Virus Material	OIU/cc	HKIU/cc	BLU/cc	ALU/cc	HKIU/ALU
Egg 72	Egg Homogenate	* 7.6	-	8.2	5.0	2.6
E56HK5	TC Fluid	5.1	6.2	-	-	-
E56HK7	TC Fluid	-	7.5	-	4.5	2.8
E56HK9	TC Fluid	-	7.0	6.2	3.9	3.1
E56HK10	TC Fluid	-	8.0	6.7	5.9	2.1

* Note: Titers are expressed as $\log_{10}$

Table 4. Mortality of adult mice inoculated with TOP strain passaged by undiluted seed for 20 times.

Passage	Death of mice inoculated i.c.					
E56HK33	*1/5	1/5	0/5	1/4	0/5	0/5
	**3.8	2.8	1.8	0.8	0.08	0.008

Note: * Number of death /Number of inoculated of mouse
** HKIU injected i.c.

Table 5. Mortality of adult mice inoculated with TOP strain during terminal dilution passages after 51st passage through one day eggs and HK cells

Passage	Death of mice inoculated i.c.					
E51	*0/5 (5.0)	0/5 (4.0)	0/5 (3.0)	0/5 (2.0)	0/5 (1.0)	0/5 (0.1)
E52	5/5 (5.8)	5/5 (4.8)	2/5 (3.8)	1/5 (2.8)	0/5 (1.8)	0/5 (0.1)
E53	2/4 (4.8)	1/5 (3.8)	1/5 (2.8)	1/5 (1.8)	0/5 (0.1)	0/5 (0.01)
E54	0/5 (5.8)	0/5 (4.8)	0/5 (3.8)	0/5 (2.8)	0/5 (1.8)	0/5 (0.1)
E54HK1	0/5 (4.0)	0/5 (3.0)	0/5 (2.0)	0/5 (1.0)	0/5 (0.1)	0/5 (0.01)
E54HK2	0/5 (4.8)	1/5 (3.8)	0/5 (2.8)	2/5 (1.8)	0/5 (0.1)	0/5 (0.01)
E54HK3	0/5 (2.8)	3/5 (1.8)	0/5 (0.1)	0/5 (0.01)	0/5 (0.001)	0/5

Note: Number of dead mice/Number of inoculated mice.
Numbers in parenthesis show HKIU inoculated i.c.

Table 6. Fate of adult mice inoculated with clones of TOP strain obtained during a few clonal passages.

1st Generation				2nd Generation				3rd Generation			
Clone No.	HKIU/cc	*Death	**Sick-ness	Clone No.	HKIU/cc	*Death	**Sick-ness	Clone No.	HKIU/cc	*Death	**Sick-ness
1-1	7.5	2/30	4/30	2-1	6.2	0/25	2/25	3-1	7.0	10/30	14/30
§ 1-2	6.7	0/25	2/25	2-2	6.3	2/25	4/25	3-2	7.3	10/30	15/30
1-3	7.7	1/30	4/30	§ 2-3	6.0	0/25	0/25	3-3	6.5	9/25	10/25
1-4	7.5	# 2/5	2/5	2-4	6.5	0/25	1/25	3-4	6.0	5/25	11/25
				2-5	6.8	9/25	13/25	3-5	6.2	5/25	9/25
				2-6	7.3	4/29	4/29	3-6	5.7	2/19	6/19
				2-7	6.5	1/25	1/25	3-7	6.5	19/24	22/24
				2-8	5.7	4/20	9/20	3-8	6.0	7/25	14/25
				2-9	6.5	7/25	9/25	3-9	6.2	7/25	11/25
								3-10	5.7	15/19	19/19
								3-11	6.7	4/25	8/25
								3-12	6.3	9/25	11/25
								3-13	5.5	4/20	8/20

* Number of dead mice/Number of mice inoculated with various dilution of virus, each containing more than 1.0 HKIU

** Numerator means number of sick mice plus number of dead mice during 3 weeks of observation. Denominator means the same thing to *

§ The population of this special clone was analysed in the following generation.

Mice inoculated only with undiluted fluid were examined.

Table 7. Effect of strain and sex of mice in regards to the susceptibility to ic challenge of TOP strain.

Virus Material	Strain of Mice	Sex of Mice	Death of mice inoculated with BLU of 4.4	3.4	2.4	1.4	0.4	in log
TOP E54HK5	GPC	Mix	*0/4	0/5	0/5	0/5	0/5	
	DD	Female	0/6	0/5	0/5	0/5	0/4	
	DD	Male	0/3	0/3	0/3	0/3	0/5	

Note: * Number of dead mice/Number of inoculated mice.

Table 8. Mortality of adult mice following ip inoculation with some strains of JBE virus.

Name of Strain	Virus Material	Mortality of mice inoculated with various dosis of BLU*						
JaTH 160	ABr-IP-5 Br.susp.	0/5 ** (6.4)	3/5 (5.4)	4/5 (4.4)	3/5 (3.4)	2/5 (2.4)	3/5 (1.4)	0/5 (0.4)
JaGAr-01-HK	HK-8 TC Fluid	0/5 (5.5)	2/5 (4.5)	2/5 (3.5)	1/5 (2.5)	3/5 (1.5)	0/5 (0.1)	0/5 (0.01)
JaGAR-02	BBr-3 Br.susp.	4/5 (9.4)	3/5 (8.4)	1/5 (7.4)	2/5 (6.4)	3/5 (5.4)	1/5 (4.4)	0/5 (3.4)
Nakayama (NIH)	ABr-? Br.susp.	1/5 (8.5)	0/5 (7.5)	0/5 (6.5)	0/5 (5.5)	0/5 (4.5)	0/5 (3.5)	0/5 (2.5)
Nakayama (Yakken)	ABr-?+88 Br.susp.	5/5 (9.3)	1/5 (8.3)	0/5 (7.3)	0/5 (6.3)	0/5 (5.3)	0/5 (4.3)	0/5 (3.3)
TOP	E54,HK-6 TC Fluid	0/5 (4.1)	0/5 (3.1)	0/5 (2.1)	0/5 (1.1)	0/5 (0.1)	0/5 (0.01)	

Note: * Numbers in parenthesis.
** Number of mice dead/Numbers of mice inoculated.

Table 9. Scheme of strain differences of JBE virus in regard to virulence to the adult mouse.

Name of Strain	Virulence determined by			
	Intraperitoneal		Intracerabral	
	Mortality	Infectivity	Mortality	Infectivity
JaTH 160 JaTH 260 JaGAr 01-HK JaGAr 02	Strong	Strong	Strong	Strong
Nakayama(NIH) Nakayama(Yakken)	Weak	Weak	Strong	Strong
TOP	Weak	Weak	Weak	Strong
Hypothetical Variant No. 1	Weak	Strong	Strong	Strong
Hypothetical Variant No. 2	Weak	Strong	Weak	Weak

Fig. 1

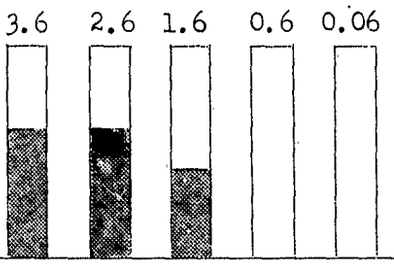
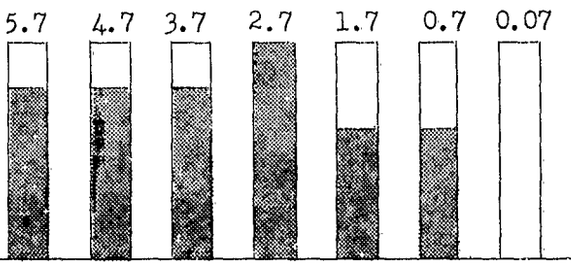
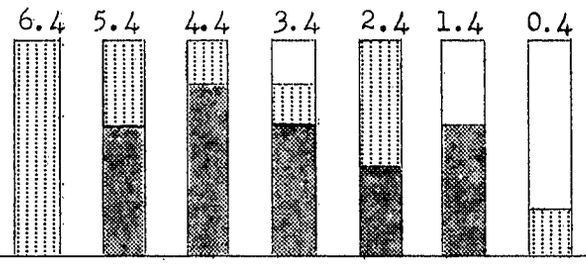
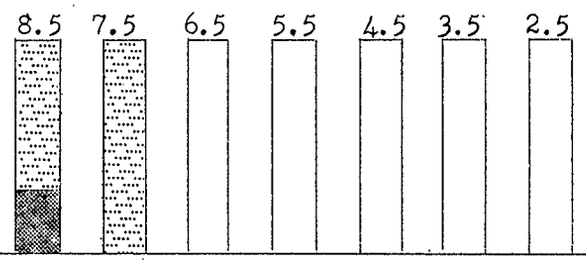
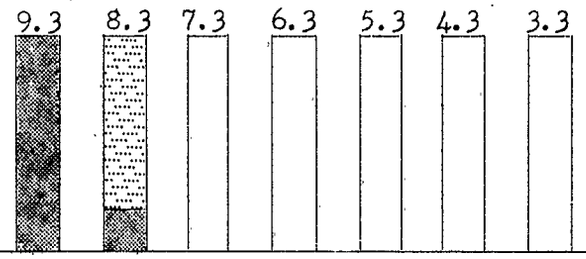
Name of Strain	Virus Material	Mortalities of mice at different dilution of virus
JaTH 160	Human Brain	
JaTH 260	Human Brain	

Fig. 3

Name of Strain	Virus Material	Death and Infection of Mice inoculated i. p.
JaTH 160	Adult Mouse Brain pass. IP-5	
Nakayama (NIH)	Adult Mouse Brain pass. Br. ?	
Nakayama (Yakken)	Adult Mouse Brain pass. Br. ?	

Note: Death Infection determined individually

Infection determined with pooled sera

Numbers on top of the column denote BLU injected.